PROTEIN KINASE Cα REQUIRED FOR VANILLOID RECEPTOR 1 ACTIVATION: EVIDENCE FOR MULTIPLE SIGNALLING PATHWAYS

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Running Title: Direct Activation of VR1 by PKCα

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SUMMARY

Activation of vanilloid receptor (VR1) with protein kinase C (PKC) was investigated in cells ectopically expressing VR1 and primary cultures of dorsal root ganglion (DRG) neurons. Submicromolar phorbol 12,13-dibutyrate (PDBu), which stimulates PKC, acutely activated Ca^{2+}-uptake in VR1-expressing cells at pH 5.5þut not at mildly acidic or neutral pH. PDBu was antagonized by bisindolylmaleimide, an PKC inhibitor, and ruthenium red, a VR1 ionophore blocker, but not capsazepine, a vanilloid antagonist indicating that catalytic activity of PKC is required for PDBu activation of VR1 ion conductance, and is independent of the vanilloid site. Chronic PDBu dramatically down-regulated PKCα, while only partially influencing PKCβ, -δ, -ε or −ζ, in DRG neurons or the VR1 cell lines. Loss of PKCα correlated with loss of response to acute re-challenge with PDBu. Anandamide, a VR1 agonist of in acidic conditions, acts additively with PKC and remains effective after chronic PDBu. This discriminates two independent VR1 activation pathways: i) direct ligand binding (anandamide, vanilloids) or ii) extracellular ligands coupled to PKC by intracellular signaling. Experiments in cell lines co-expressing VR1 with different PKC isozymes showed that acute PDBu-induced activation requires PKCα , but not PKCε. These studies suggest that PKCα in sensory neurons may elicit or enhance pain during inflammation or ischemia.

INTRODUCTION

Vanilloid receptor type 1 (VR1) is expressed almost uniquely in small size dorsal root ganglia neurons, which are involved in transmitting noxious heat and chemical stimuli from the periphery. Pungent vanilloids, such as capsaicin (CAP) or resiniferatoxin (RTX) (1-4), and eicosanoids, combined with low extracellular (pH < 6.5) (5,6) can activate VR1-positive neurons cultured from dorsal root ganglia (DRG), and cells ectopically expressing VR1 (5). In addition, evidence is accumulating that protein kinases, including PKA and PKC may directly induce or potentiate VR1 activity (7-9). However, the specific PKC isozyme(s) and exact mechanism(s) leading to channel opening are under active investigation. Acute application of phorbol esters sensitizes knee joint sensory nerves (10), and enhances neuronal ionic currents activated by a noxious thermal stimulus (11,12), but these studies did not connect PKC activation to VR1 activation. PKC also seems to be an important mediator of bradykinin (BK)-, and epinephrine-induced hyperalgesia and nociceptor sensitization (13-15).

Thus, PKC appears to be an effector in multiple signal transduction cascades in primary afferent neurons. However, PKC is not a "single entity", but a family of at least 11 serine-threonine kinases with different protein substrate specificities (16-18). While there are functional differences between PKC isozymes, it is not

exactly known which isozymes contribute to nociceptive processing. One approach to demonstrate specific involvement of a particular PKC isozyme is targeted gene deletion. Knockout of the PKCγ isozyme in mice suggested that this enzyme, which is normally highly expressed in specific second order neurons in spinal lamina IIi, was involved in the proper processing of incoming afferent nociceptive signals (19-22).

Another approach to identify specific isozyme participation is selective down-regulation of PKC isotypes by exposure to active phorbol esters. Acute treatment (i.e. 1 μM PDBu for 10 min) not only activates classical and novel PKC isozymes but also induces enzyme translocation to the plasma membrane. Upon chronic exposure (~24 hrs) this leads to isozyme selective proteolysis and differential down-regulation. Either classical PKC isotypes, activated by diacylglycerol (DAG) and Ca^{2+}, or novel PKC isotypes, activated by DAG only, can be specifically down-regulated by micromolar doses of PDBu, but to different degrees (23). In primary sensory neurons Cesare et al. reported expression of only 5 isoforms, PKCβ_I, -β_{II}, -δ, -ε, and –ζ (12). PKCγ, a previously reported pain related isozyme in the spinal cord, was apparently not readily detected in DRG cultures prepared from newborn rats (12,20). Upon treatment with BK, an endogenous algesic peptide, only PKCε was determined to translocate to the plasma membrane in DRG cultures prepared from 2 days old rats (12). In contrast

to the isozyme pattern detected in sensory neurons from newborns, PKCα, an additional isozyme, was positively identified in DRG cultures prepared from 3-month-old rats, which may regulate VR1 in adults (24). BK was also found to release VR1 from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2)-mediated inhibition, a mechanism proposed for pain-specific channel opening (25).

The intracellular signaling pathways upstream of PKC isozymes in nociceptive afferents still represent open questions. Receptors for histamine, peptides, different allergens, and neuronal growth and survival factors and eicosanoids, which are connected *via* phospholipases (i.e. PLC and PLD) to PKC signaling, are all good candidates to modulate VR1 activity of sensory neurons. Nerve growth factor (NGF), abundantly synthesized *de novo* after spinal cord injury and inflammation, was noted to couple to PKC. Administration of NGF to neonatal and adult rats *in vivo* was reported to produce hyperalgesia through activation of PKC (26). Axonal growth and differentiation experiments in cultured PC-12 rat pheochromocytoma cells, pointed toward the novel PKCε, a Ca^{2+}-insensitive isotype, as an effector enzyme for NGF-mediated intracellular signaling (27,28). NGF pretreatment also was noted to potentiate CAP-induced inward currents in voltage clamped DRG neurons, suggesting either synergy or additivity (29). Moreover, other neurotrophins (NT), such as NT-3 can influence the central terminals of their respective afferent neurons after injury (30). Among several

mRNAs, NGF can induce transcription of VR1 in dissociated cultures of adult DRG, as well as the VR1 protein levels in the undamaged DRG neurons after partial nerve injury (31,32). Together these observations suggest that NGF may engender hyperalgesia either by *de novo* transcription and translation or by direct, PKC-mediated activation of VR1. The regulation of VR1 by PKC is also supported by the PKCε−mediated increase in VR1 ion currents in DRG neurons and in cells ectopically expressing recombinant vanilloid receptor (8). In addition or as an alternative to direct signaling *via* PKC, NGF was observed to act as a liberator of VR1 from PtdIns(4,5)P_2-mediated blockade (25).

In the current study, we present evidence suggesting a direct role of PKC in VR1 activation. In particular, full expression of PKCα was found to be essential for acute activation of VR1 with PDBu, a synthetic, stable agonist of classic and novel PKCs. This action was examined in primary DRG cultures as well as NIH 3T3 and HeLa cells stably transfected to express VR1ε, a C-terminal ε-epitope tagged VR1 recombinant (4). Each system has a different complementary composition of PKC isozymes, which permitted discrimination of the isoforms that affected VR1 function. NIH 3T3 cells were determined to express classical PKCα novel PKCδ, -ε, and atypical -ζ isoforms, respectively (33). In contrast, PKCε, a previously proposed up-stream regulator of VR1 was not expressed in HeLa and was not required for activation of ectopically expressed VR1. Our findings in

DRG cultures, VR1ε-NIH 3T3 and VR1ε-HeLa cell lines, strongly suggest that PKCα can sensitize and/or activate VR1 in the absence of endogenous ligands, including anandamide (ANA), an endovanilloid/eicosanoid agonist of VR1 in acidosis (5). In VR1ε-HeLa cells, another permanent line we established, acute PDBu induced ionophore activity of VR1 even in the absence of PKCε. ANA activates VR1 in an additive fashion with PKC, yet is independent of PKC, since ANA activation is retained after chronic PDBu-mediated down regulation of PKCα. Similar to ANA (5), the PKC activation of VR1 requires a drop of extracellular pH < 6.5, indicating that the protonated VR1 conformer serves as a better substrate of PKC. Our observations emphasize the conditional requirements for the actions of endovanilloids and PKC and their capacity to work in a concerted, additive fashion on the nociceptive nerve terminal.

EXPERIMENTAL PROCEDURES

Preparation of VR1ε expressing NIH 3T3 cell line: C-terminally tagged chimeric rat VR1ε was prepared in the pεMTH vector plasmid vector (34), as described previously (4). To develop cell lines permanently expressing VR1ε, we used mouse NIH 3T3 fibroblasts and human HeLa adenocarcinoma-derived cells. The parental lines were determined to have low levels of Ca^{2+}-transport that were not altered by treatment with either vanilloids, ANA or PDBu. To avoid toxicity

that occurs with VR1 over-expression, VR1ε was expressed in the cell lines using only the basal activity of the metallothionein promoter, as described earlier (5). For the purpose of clarity, in sentences where PKCε is discussed in conjunction with VR1ε (e.g. VR1ε-HeLa) we refer to it as VR1 (e.g. VR1-HeLa).

DRG culture: DRG neuron enriched cultures were prepared from embryonic rats (E16) (4). Briefly, DRGs were dissected and then processed in fresh dissection medium (Lebowitz Medium, Life Technologies) until plated in DMEM. The DMEM contained 20 mM HEPES (to prevent acidification and stabilize pH at 7.4), 7.5% fetal bovine serum, 7.5% horse serum, 5 mg/ml uridine supplemented with 2 mg/ml FUDR and 40 ng/ml NGF to inhibit cell division and to promote neuronal survival and differentiation, respectively. Surfaces for cell culture were coated with poly-D-lysine and laminin. Cells were seeded on 25-mm glass coverslips or on multi-well microtiter plates. Cultures were selected in this medium for 1 week, at which point well-differentiated neurons dominated the population. Primary DRG cultures in this stage were used in radioactive and fluorescent video microscopy Ca^{2+} uptake assays.

Ca^{2+}-transport: $^{45}Ca^{2+}$-uptake experiments were carried out on one week old primary DRG cultures ($3x10^4$ cells/well) and on established VR1ε-NIH 3T3 and HeLa cell lines ($3x10^4$/well), which were seeded one day before use. Immediately before the assay, cells were adapted to room temperature (24 ˚C) for 5 min in

Hanks' balanced salt solution (pH = 6.0), supplemented with 10 µM Ca^{2+} and 0.1 mg/ml bovine serum albumin (HCB). $^{45}Ca^{2+}$-uptake was performed for 10 min at 24˚C in HCB using 0.2 µCi $^{45}Ca^{2+}$ as radioactive tracer in a 200 µl final volume. To determine the pH dependency of the $^{45}Ca^{2+}$-uptake, HCB was buffered with 20 mM Tris-HCl, adjusted to the indicated pH with 1M 2-[N-Morpholinoethanesulfonic acid (MES) (HCBTM). To stop $^{45}Ca^{2+}$-uptake, cells were rapidly changed back into 1 ml HCB, washed two additional times with 1 ml HCB, and then lysed in 200 µl/well RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA) for 30 min. Aliquots of the solubilized cell extracts were counted in a liquid scintillation counter.

Fluorescent video microscopic determination of $[Ca^{2+}]_i$ – For determination of $[Ca^{2+}]_I$ an Olympus BX 60 microscope equipped with a low-light fluorescence intensifier system was used, as described earlier (35). DRG cultures were pre-loaded with 5 µM fluo-4 AM dye for 30 min at 34˚C, and then washed three times in HCB to remove excess dye and kept in the dark for at least 15 min before starting the experiments. Recordings were carried out in a closed imaging chamber (PH-2, Warner Instrument Corp., Hamden CT), which was perfused with a pump (Miniplus 3, Gilson, France). The emitted fluorescence intensity was calculated from images taken at 10 sec intervals by the SYNAPSE 3.6e software, an image

acquisition and analysis program, as recommended by the manufacturer (Synergy Research, Silver Spring, MD). To monitor vanilloid induced changes in $[Ca^{2+}]_I$, after 1 min of baseline recording, DRG neuron cultures were perfused with 1 μM CAP in HCBTM for 30 sec, then the medium was changed back to drug free HCBTM.

Preparation of VR1 specific antibody and Western blotting- The hydrophilic C-terminal fragment of rat VR1 (M682-K838), extended with a C-terminal $(His)_6$ tag was amplified by the polymerase chain reaction employing GGAAGGATTTCAGAATTCATGGGTGAGACCGTCAACAAGATT as forward primer and TGTCGACCTCGACTCAATGATGATGATGATGATGTTTCTCCCCTGGGAC CATGGAATC as reverse primer. The PCR amplified cDNA fragment incorporated the Eco RI and Sal I restriction sites from the forward and reverse primers, respectively, and these sites were used to insert the fragment into the pMALc2x (New England BioLabs) prokaryotic expression vector. The peptide fragment was cloned in-frame and down-stream of the coding region for maltose binding protein (MBP). E. *coli* cells were transformed with the MBP-VR1C$(His)_6$ construct and production of the fusion protein was induced by 1 mM IPTG for 3 hrs. The MBP-VR1C$(His)_6$ chimeric protein was double affinity purified using the MBP and His tags, consecutively, as recommended by the manufacturers (New

England BioLabs, Novagen). The affinity purified MBP-VR1C$(His)_6$ protein was used to immunize two rabbits. After 8 weeks of immunizations at biweekly intervals, sera were tested at 1:500 dilutions in Western blotting experiments using SDS extracts prepared from neuron-enriched DRG cultures of E16 embryonic rats. The immune sera recognized a protein band at 92 $\pm$ 4 kDa in DRG extracts similar to the theoretical molecular weight of rat holo-VR1 (94,943 Da) (36).

Materials – ANA (1:4 in a soy bean oil:water emulsion), was purchased from Tocris and PDBu from Calbiochem. ^{45}Ca was purchased from ICN. PKC isozyme specific antibodies were obtained as follows: PKCα, clone M6 mouse monoclonal from Upstate Biotechnology, PKCδ and -ε affinity purified polyclonals from Life Technologies and PKCζ from Calbiochem. Affinity purified anti-RAKIGQGTKAPEEKTANTISK, a specific PKCβ peptide antibody was prepared in rabbits and characterized as described earlier (37).

RESULTS

To study PKC-dependent up-regulation of VR1, permanent cell lines ectopically expressing an ε-epitope tagged recombinant of VR1 were established in both NIH 3T3 cells (4) and HeLa cells. VR1 specificity and pH-dependency of acute (10 min) PDBu-induced $^{45}Ca^{2+}$-transport were studied in the parental, immortalized NIH 3T3 mouse fibroblast and VR1ε-NIH 3T3 cell lines at pH 5.5

and 7.5, as indicated (Fig. 1). In the parental line, the basal Ca^{2+}-uptake at pH 7.5 was about 4 times higher than at 5.5, and addition of PDBu to the assay medium had little or no effect on the basal uptake at either pH. Expression of recombinant VR1 elevated the baseline compared to parental NIH 3T3 cells at either pH 5.5 or 7.5. At pH 6.5, 7.0, and 7.5 only minor increases in calcium uptake occurred with ascending PDBu concentrations. However, the net change for VR1ε-NIH 3T3 cells was not different from the parental cell line at pH 7.5 (open triangles), indicating that these elevations are not dependent on the expression of VR1. The marked transition in ionophore activity occurs with PDBu treatment when the VR1ε-NIH 3T3 cells are at pH 5.5. Addition of PDBu at sub-micromolar concentrations increased Ca^{2+}-transport in a concentration-dependent manner between 3- and 4-fold over baseline as determined in repeated experiments (Figs. 1 and 2). An increase in Ca^{2+}-uptake could be detected at around 20 nM and the EC_{50} of PDBu-induced $^{45}Ca^{2+}$-uptake at pH 5.5 was ~100 nM, and reached a plateau at concentrations above 1 μM (Fig. 1 and 2*a*).

To further study the PKC specificity of PDBu-induced, VR1-mediated $^{45}Ca^{2+}$-uptake at pH 5.5, bisindolylmaleimide (BIS), an inhibitor directed to the catalytic ATP binding site of PKC, was co-incubated with increasing concentrations of PDBu (Fig. 2*a*). BIS (25 μM) added together with progressively increasing concentrations of PDBu completely inhibited the inducible portion of

the $^{45}Ca^{2+}$-uptake, but had only slight or no effect on baseline uptake in VR1ε-NIH 3T3 cells. Studies with VR1 selective inhibitors showed that acute PDBu-induced $^{45}Ca^{2+}$-transport were nearly completely inhibited by 10 μM RR (a blocker of the VR1 ionophore), but not with a high concentration (25 μM) of capsazepine (CPZ, a competitive antagonist at the vanilloid ligand binding site) (Fig. 2*b*).

Western blotting with specific antibodies was employed to analyze expression of VR1 and different PKC isozymes in DRG cultures and in VR1ε-NIH 3T3 cells (Fig. 3). The DRG cultures from E16 embryonic rats were enriched for neurons by a one-week treatment with FUDR to eliminate dividing cells. Cultures were also treated with NGF to promote neuronal differentiation. Before Western blotting, the DRG cultures were morphologically and functionally characterized with phase contrast and fluorescence video microscopy. After 1 week, the DRG cultures typically contained large (25-30 μ in diameter), medium (~15-25 μ), and small size (7-10 μ) neurons in close to equal ratio. The CAP-induced increase of intracellular $[Ca^{2+}]_i$ in the DRG cultures is one of the markers we used to identify of VR1-expressing nociceptors. After loading cultures with fluo-4, a Ca^{2+} sensitive fluorescent dye, the cells within the later two populations (~30% of the total) responded to 1 μM CAP with elevated green fluorescence (Fig. 3*a* vs. *b*).

VR1 expression was determined immunochemically by a rabbit antibody, raised against MBP-VR1CHis, a soluble, C-terminal fragment (M682-K838) of rat

VR1. The immune sera recognized a prominent band at 92 $\pm$ 4 kDa in DRG extracts (Fig. 3*c*), which corresponds well with theoretical molecular weight of rat holo-VR1 (94.9 kDa). In addition to the 94.9 kDa protein, our antibody detected a 114 kDa glycosylated form of VR1 (36), in extracts from VR1ε-NIH 3T3 cells (Fig. 3*d*). The expression of different isoforms of PKC in extracts prepared from either control or chronic PDBu treated DRG cultures or VR1ε-NIH 3T3 cells was determined by Western blotting employing isotype-specific antibodies. The DRG cultures expressed PKCα, in addition to PKCβ, -δ, -ε, and -ζ isozymes; the latter have been reported previously in cultures from neonatal animals (12). Chronic PDBu treatment had the most dramatic effect on the PKCα isozyme, which was almost completely down-regulated in both DRG neurons and VR1ε-NIH 3T3 cells (Fig. 3 *c* and *d*). However, chronic PDBu produced only a partial down-regulation of the PKC β, -δ, -ε isoforms in DRG neurons. Similar effects were seen in the VR1ε-NIH 3T3 cells (Fig. 3*c* and *d*) except that no PKCβ was measured which is in accordance with previous observations in the parental NIH 3T3 cell line (33) (Fig. 3*d*). As expected from a non-phorbol binding isotype, little or no change was noted in the levels of PKCζ in either cell system.

The effects of chronic PDBu-induced down-regulation on $^{45}Ca^{2+}$-uptake were studied in a parallel set of DRG cultures as those analyzed above for VR1 and PKC isozyme expression. Chronic PDBu almost completely eliminated the acute

PDBu-inducible activity as assayed by a short, 10 min re-exposure to PDBu in the presence of $^{45}Ca^{2+}$ (Fig. 4*a*). Elimination of the acute PDBu effect correlated with the complete down-regulation of PKCα in DRG cultures chronically treated with PDBu (Figs. 3*c* and 4*a*). In control DRG cultures, addition of ANA, a vanilloid ligand in acidic conditions, induced $^{45}Ca^{2+}$-uptake at pH 5.5 with similar kinetic parameters as previously characterized (5). After chronic PDBu treatment, the ANA-induced Ca^{2+}-transport remained intact, although shifted slightly into a lower affinity region; an EC_{50} of ~20 μM compared to ~10 μM, was obtained in the experiment shown (Fig. 4*b*). Parallel experiments repeated in VR1ε-NIH 3T3 cells also resulted in complete inhibition of acute PDBu-induced $^{45}Ca^{2+}$-uptake (Fig. 5*a*), but left the ANA-elicited $^{45}Ca^{2+}$-transport intact (Fig. 5*b*).

Activation of VR1 by the endovanilloid ANA and PKC activator PDBu appear to represent two independent and potentially additive mechanisms. The inhibition studies in Figure 2*b* show that acute PDBu-induced $^{45}Ca^{2+}$ transport is not blocked with 25 μM CPZ, a competitive vanilloid antagonist, but is blocked with 10 μM RR, which inhibits Ca^{2+} entry through the VR1 ion channel (38). The VR1 specificity of the $^{45}Ca^{2+}$-uptake was analyzed by co-application of either 25 μM RR or 10 μM CPZ at ascending concentrations of ANA at pH 5.5. Either RR or CPZ eliminated the ANA inducible portion of $^{45}Ca^{2+}$-uptake, indicating that ANA has to specifically interact with VR1 to induce cation transport (Fig. 6). Co-

application of 1 μM PDBu with each concentration of ANA shifted the dose-response curve to left and augmented $^{45}Ca^{2+}$-uptake in a manner characteristic of additive and independent routes for VR1 activation (Fig. 6).

A previous study suggested PKCε as a unique upstream regulator of VR1 in DRG nociceptors (8). The present experiments suggest rather that PKCα is required for intracellular activation of VR1 in DRG neurons. A remarkable correlation between down-regulation of PKCα and loss of acute PDBu-induced, VR1-mediated Ca^{2+}-transport was determined both in embryonic rat DRG cultures and in VR1ε-NIH 3T3 cells. To examine the requirement for PKCε in VR1 activation, a permanent VR1-expressing HeLa cell line (VR1ε-HeLa) was established. This cell line exhibits a different PKC isozyme composition (notably PKCε is not expressed) (39) than either the NIH 3T3 cells or the DRG neurons. The HeLa line stably expressing VR1 showed approximately a 10-fold elevation of $^{45}Ca^{2+}$-uptake above baseline when treated with a maximal dose of CAP (10 μM) or RTX (10 nM) at pH 5.5 (Fig. 7*a*). We corroborated the lack of PKCε in the VR1 expressing HeLa cells (39), with isozyme-specific Western blots (Fig. 7*b*). Similar to parental cells, the VR1-HeLa subclone expresses PKCα, −β, -δ, and -ζ , but not -ε (Fig. 7*b*). As seen in DRG neurons and NIH 3T3 cells, chronic treatment with PDBu, almost completely down-regulated PKCα from VR1−expressing HeLa cells. At the same time little or no change in the levels

PKC β, -δ, and -ζ or VR1 were detected (Fig. 7). Coincident with the loss of PKCα, the VR1-expressing HeLa cells exhibited inhibition of acute PDBu-induced $^{45}Ca^{2+}$-uptake (Fig. 8*a*) without and significant impact on Ca^{2+}-uptake induced by the VR1 agonist ANA (Fig. 8*b*). As an aside, we note that the VR1-expressing HeLa line showed about 5-fold higher affinity toward ANA and high concentrations of ANA (above 10 μM) caused an inhibition of $^{45}Ca^{2+}$-uptake, determined at pH 5.5, which was not seen in VR1-expressing NIH 3T3 cells (Figs. 5*b*, 6 *vs.* 8*b*).

DISCUSSION

PKC mediated up-regulation of VR1 was investigated in DRG neurons, NIH 3T3 and HeLa cell lines, with the latter two permanently expressing VR1ε, a C-terminal ε-epitope tagged recombinant of rat vanilloid receptor type 1. We observed that PDBu, a synthetic agonist capable of binding to the regulatory domain of classical and novel PKC isozymes, activated Ca^{2+}-uptake in the absence of any added vanilloid ligand in all three cell preparations. PDBu-evoked channel activation exhibited a strong pH dependency: VR1ε-NIH 3T3 and VR1ε-HeLa cells responded to less than 1 μM PDBu at acidic pH (pH 5.5), but not at pH 6.5 or higher (up to pH 7.5). We previously observed that ANA activated VR1 $^{45}Ca^{2+}$-uptake in cells ectopically expressing VR1 or in primary DRG cultures only when

the assay media was acidic (pH < 6.5) (5). Protonation of VR1 in an acidic environment does not directly cause channel opening (6) or Ca^{2+}-uptake (Fig. 1), but increases the affinity of the receptor for vanilloid and eicosanoid ligands (5). This observation prompted us and others to suggest that VR1 exists in two distinct conformations with H^+ serving as an allosteric switch (5,40,41). Between pH 6.5 – 7.5, protons dissociate from VR1, perhaps in the pore loop region (42,43), yielding a conformer with lower affinity toward vanilloids and vanilloid-like eicosanoids such as ANA (5,6). However, the protonated form of VR1 (between pH 6.5 – 5.5) displays an increased affinity toward vanilloid ligands, which coincides with a decreased capacity for cation transport (5). The fact that PDBu induces $^{45}Ca^{2+}$-transport through VR1 when the medium is acidic is consistent with the hypothesis that the protonated conformer of VR1 is preferred as the substrate of PKC. Sequence analysis of rat and human VR1 indicates 18 intracellular sites that conform to PKC consensus motifs for potential phosphorylation. This highlights the need for determining the exact phosphorylation site(s) involved in VR1 regulation.

Studies with selective inhibitors of VR1 showed that PDBu can induce $^{45}Ca^{2+}$-transport in the presence of 25 µM CPZ, a competitive inhibitor of vanilloid ligand binding (Fig. 2*b*). In contrast to CPZ, RR, a blocker of the VR1 ion channel (38) can prevent $^{45}Ca^{2+}$-uptake activated by both vanilloid agonists or PKC (Figs

2*b* and 6). In addition, results with BIS indicate that the catalytic activity of PKC is required for VR1-mediated $^{45}Ca^{2+}$-uptake (Fig. 2). BIS eliminates the effect of acute PDBu when they are co-applied to VR1ε-NIH 3T3 and VR1ε-HeLa cells, suggesting that phorbol esters activate VR1 only indirectly, *via* intact catalytic activity of PKC. In contrast to a previous report (25), but in concert with findings of others, our PKC down–regulation experiments also indicate that PDBu does not directly interact with VR1 (44-46). However, from the literature, it was not clear which isozymes are expressed and mediate VR1 activation in sensory ganglia neurons. Data from different studies are not completely in accord, consequently, different conclusions about which PKC isozyme regulates VR1 have been reached (12,20,24). In our DRG cultures the presence of VR1 was detected with physiological methods using a CAP-induced increase in $[Ca^{2+}]_i$, as measured by fluo-4, and biochemically by Western blotting. In addition to VR1, among the PKC isoforms, we detected the presence of PKCα a result similar to that reported for 3-month-old rats (24), which is in contrast to what was reported for cultures prepared from 2 days old animals (12). The ability to positively detect PKCα may be related to differences in antibodies used or in methodology of the primary culture systems (Fig. 3).

The results of the chronic PDBu down-regulation experiments support a key role for the PKCα isozyme in activation of VR1. Following chronic PDBu

treatment (24 hrs, 2 μM PDBu), PKCα down-regulated almost completely in DRG neuron cultures and the NIH 3T3 and HeLa cell lines ectopically expressing VR1, while the levels of other isozymes either did not change, or decreased only partially. The loss of PKCα correlated with loss of the acute activation by PDBu on $^{45}Ca^{2+}$-uptake in all three cell preparations. However, direct activation through the vanilloid agonist route remained intact since stimulation by ANA exhibited little alteration (Figs. 4, 5 and 8). These experiments indicate an important role of PKCα isozyme on VR1 function through intracellular signaling pathways. Previously HeLa, a human carcinoma cell line was found not to express PKCε (39) and we confirmed its absence in our clonal VR1-HeLa cell line by Western blot analysis (Fig. 7). Although PKCε has been proposed as an upstream regulator of VR1 by others (8,15,47), the expression of PKCε does not appear to be a necessary component for VR1 activation in VR1-HeLa cells by acute PDBu (Fig. 8*a*). In contrast, down-regulation of PKCα (Fig.7.) dramatically reduced the effect of PDBu in the VR1−HeLa line (Fig. 8*a*), again emphasizing an essential role for PKCα in VR1 activity.

Taken together, these data suggest that, in addition to heat, two independent signaling pathways can regulate the ionophore function of VR1 and hence nociceptive and inflammatory pain signaling. The first pathway utilizes vanilloid ligands or eicosanoid/endovanilloid/endocannabinoid compounds such as ANA,

leukotriene B(4), 12- and 15-(S)-hydroperoxyeicosatetraenoic acids, 5- and 15-(S)-hydroxyeicosatetraenoic acids (5,6). The second pathway uses PKC, and likely functions as an intracellular second messenger-coupled effector for extracellular inflammatory agents. Both pathways are conditionally dependent on pH and we hypothesize that the protonated form of VR1 is the preferred substrate for PKC. The two pathways produce additive effects on VR1-mediated $^{45}Ca^{2+}$-uptake. However, they can be dissociated, as shown by the selective effect of chronic PDBu down-regulation on acute activation of VR1: ANA/vanilloid signaling is preserved whereas signaling through the PKC pathway is eliminated. Thus, intracellular signaling by PKC isozymes is not necessary for ligand-induced, direct activation of VR1, but PKC isozymes can open the VR1 ionophore in the absence of extracellular vanilloid-like ligands. The importance of the PKCα isoform in VR1 regulation, in comparison to the ε isoform, was evident in chronic PDBu down-regulation experiments carried out with VR1-NIH 3T3 cells and DRG neurons. Furthermore, in the VR1-HeLa cell line, acute PDBu was as effective as in our other VR1 expressing preparations, despite its lack of the PKCε isoform.

ANA and PDBu co-incubation experiments highlights the independence of the two pathways as well as the potential for mutual interaction of nociceptive stimuli to reinforce and sustain pain signaling. The number of different signal transducers that are upstream of different PKC isozymes in primary afferent

endings is still an open question. Various agonists implicated in pain, including NGF, BK, and SP have receptors in nociceptors and are coupled to different PLC and PLD isozymes. These lipases, upon activation, produce diacylglycerol (DAG) at the plasma membrane (18,48,49) which is able to activate PKC generally, and in particular the classic PKCα in sensory neurons, as suggested here, or novel PKCε isotypes proposed previously by others (12,15,28,47,50).

In conclusion, PKC-induced activation of VR1 is a novel regulatory pathway, which deserves further investigation. Similar to endovanilloid ligand-induced ionophore activity of VR1, a drop in pH below 6.5 is necessary for PDBu-evoked calcium uptake. PKCε was proposed previously as one of the likely isozymes to activate VR1 by transmembrane signaling. Here we positively identify PKCα, in thoroughly characterized DRG neuron cultures from embryonic rats, as a necessary factor for acute PDBu-induced activation of VR1. The PKCε isoform does not appear to be absolutely necessary for VR1 activation as shown by VR1-HeLa, a permanent cell line not expressing PKCε. An interesting aspect of PKC isozyme-specific regulation of VR1 is that certain isotype(s) may be therapeutic targets for novel pain treatments. Specific inhibition or elimination of PKCα in the periphery may result in effective pain management in nerve injuries where upstream regulators of PKC isozymes are overproduced and may contribute to intractable pain states.

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(i) FOOTNOTES

[1]The abbreviations used are: CAP, Capsaicin; CPZ, capsazepine; RTX, resiniferatoxin; DRG, dorsal root ganglion; TG, trigeminal ganglion; ER, endoplasmic reticulum; $[Ca^{2+}]_i$, intracellular free calcium; VR1, vanilloid receptor; VR1ε, C-terminally ε-tagged vanilloid receptor, NGF, neuronal growth factor; FUDR, 5-fluoro-2'-deoxyuridine; DMEM, Dulbecco's Modified Eagle Medium; HEPES; N-2-Hydroxyethylpiperazine-N´-2-Ethane Sulfonic Acid. MES, 2-[N-Morpholino]ethanesulfonic acid.

FIGURES

Fig. 1. Effect of pH on VR1-mediated Ca^{2+}-transport. NIH 3T3 and VR1ε-NIH 3T3 cells, a permanent cell line ectopically expressing an ε-epitope tagged recombinant of VR1, were seeded in 96 well plates ($3x10^4$ cells/well), one day before the experiments. $^{45}Ca^{2+}$-uptake assays were carried out as described in the Experimental Procedures. At pH 5.5 the basal Ca^{2+}-uptake was lower than at pH

7.5, but PDBu clearly activated Ca^{2+}-transport in a dose-dependent manner (2-4 fold determined in independent experiments), and reached saturation at 1 μM (EC_{50} ~ 150 nM). In contrast, no strong induction with PDBu was noted in the parental NIH 3T3 cells at either pH 5.5 or 7.5. In comparison to the parental cells at pH 7.5, PDBu failed to produce a net activation of Ca^{2+}-uptake at pH 6.5, 7.0, and 7.5 in the stable VR1-expressing cells. Each point on the graph is the average of triplicate determinations. Experiments were repeated two additional times in triplicate with similar results.

Fig. 2. PDBu and PKC catalytic activity and ion conductance of VR1. (*a*) Inhibition of PDBu-induced Ca^{2+}-uptake was analyzed at pH 5.5 with bisindolylmaleimide (BIS), an ATP binding site directed inhibitor of PKC. BIS (25 μM) added together with increasing concentration of PDBu completely inhibited the PDBu-inducible portion of $^{45}Ca^{2+}$-uptake in VR1ε-NIH 3T3 cells, indicating that catalytic activity of PKC is required for up-regulation of the VR1 channel. Each point represents the mean $\pm$ SE of triplicate determinations. Similar results were obtained in two independent experiments also performed in triplicate. (*b*) PDBu-activated calcium uptake was inhibited by ruthenium red (RR) a VR1 channel blocker, but not by capsazepine (CPZ), an antagonist at the vanilloid

binding site. Similar results were obtained in two additional experiments carried out in triplicates.

Fig. 3. PKC down-regulation. Chronic (2 μM, 24 hrs), PDBu-induced down-regulation of PKC isozymes was determined by Western blotting in DRG cultures and NIH 3T3 cells expressing VR1ε. (*a*) DRG cultures were prepared as described and loaded with fluo–4 AM and (*b*) treated with 1 μM CAP for 15 sec. About 30 % of the neuronal perikarya responded with intense fluorescence indicating expression of VR1 (white arrows). (*c*) Cultures (~250,000 neurons) were extracted in hot-SDS sample buffer and proteins were size separated by 10 % PAGE, then electroblotted to nitrocellulose membranes. Levels of PKC isozymes were determined by isotype-specific antibodies before and after chronic PDBu treatment (24 hrs). VR1 was determined by a rabbit antibody raised against the affinity purified MBP-VR1C-$(His)_6$ antigen, as described. Baseline DRG cultures expressed VR1 and PKCα, -β, -δ, -ε, and -ζ isozymes. Chronic PDBu down-regulated PKCα almost completely, however, the levels of PKCβ, -δ, -ε, and VR1 were only partially affected. (*d*) The same antigens were analyzed in VR1-NIH 3T3 cell lines. In contrast to DRG, PKCβ is not expressed in VR1-NIH 3T3 cells. Similar to the DRG, ~90 % loss of PKCα was detected with only minor effects, if any, on the other isozymes. Levels of PKCζ did not change in either DRG or

VR1-NIH 3T3 cells which served as an internal control for protein loading. Star stands for glycosylated form (110 kDa) of VR1 detected in cell lines ectopically expressing VR1. Similar results were obtained in a second, independent experiment.

Fig. 4. DRG neurons. The effects of chronic (2 μM, 24 hrs) PDBu treatment were studied on the same batch of DRG neurons analyzed before for VR1 and PKC isozymes with Western blotting. (a) Chronic treatment completely inhibited the acute effect of PDBu on VR1-mediated $^{45}Ca^{2+}$-uptake. (*b*) At the same time the agonist actions of ANA are preserved, although the affinity of ANA for VR1 for is reduced (non-treated EC_{50} ~7 μM vs. ~25 μM, after chronic PDBu). Similar results were obtained in a second independent experiment.

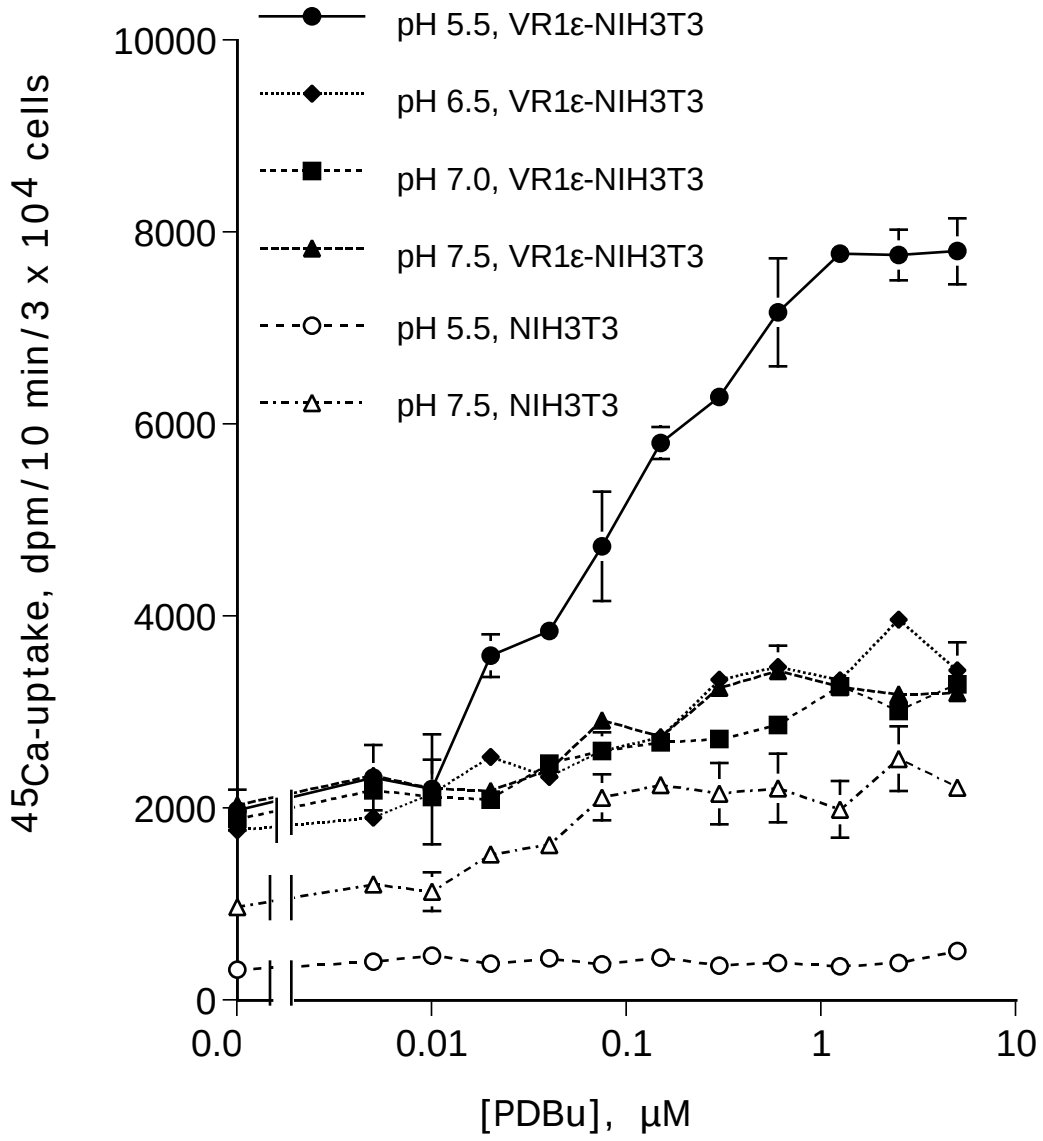
Fig. 5. VR1ε-NIH 3T3 cells. Effect of chronic (2 μM, 24 hrs) PDBu treatment was studied on the same batch of VR1ε-NIH 3T3 cells analyzed for VR1 and PKC isozymes with Western blotting. (*a*) Chronic exposure completely inhibited the acute effect of PDBu on VR1-mediated $^{45}Ca^{2+}$-uptake in NIH 3T3 cells expressing VR1. (*b*) Chronic treatment did not affect ANA-induced $^{45}Ca^{2+}$-transport. Similar results were obtained in a second independent experiment.

Fig. 6. ANA and acute (10 min, 1 μM) PDBu activate VR1 calcium flux in an additive manner. Experiments were carried out in VR1ε-NIH 3T3 cells in acidic assay medium (pH 5.5). The maximum effect of ANA induced $^{45}Ca^{2+}$-uptake ~5 times above the baseline at pH 5.5. An additive effect was seen with co-incubation of 1 μM PDBu and increasing concentrations of ANA (filled triangles). The addition of PDBu elevated the V_{max} of Ca^{2+}-transport, and shifted EC_{50} of ANA from ~10 to ~4 μM in VR1ε-NIH3T3 cells. As expected, capsazepine (CPZ), a competitive inhibitor of vanilloid ligand binding, and ruthenium red (RR) a selective channel blocker of VR1 almost completely inhibited ANA action. Each point represents the mean $\pm$ SE of triplicate determinations. Similar results were obtained in two independent experiments also performed in triplicate.

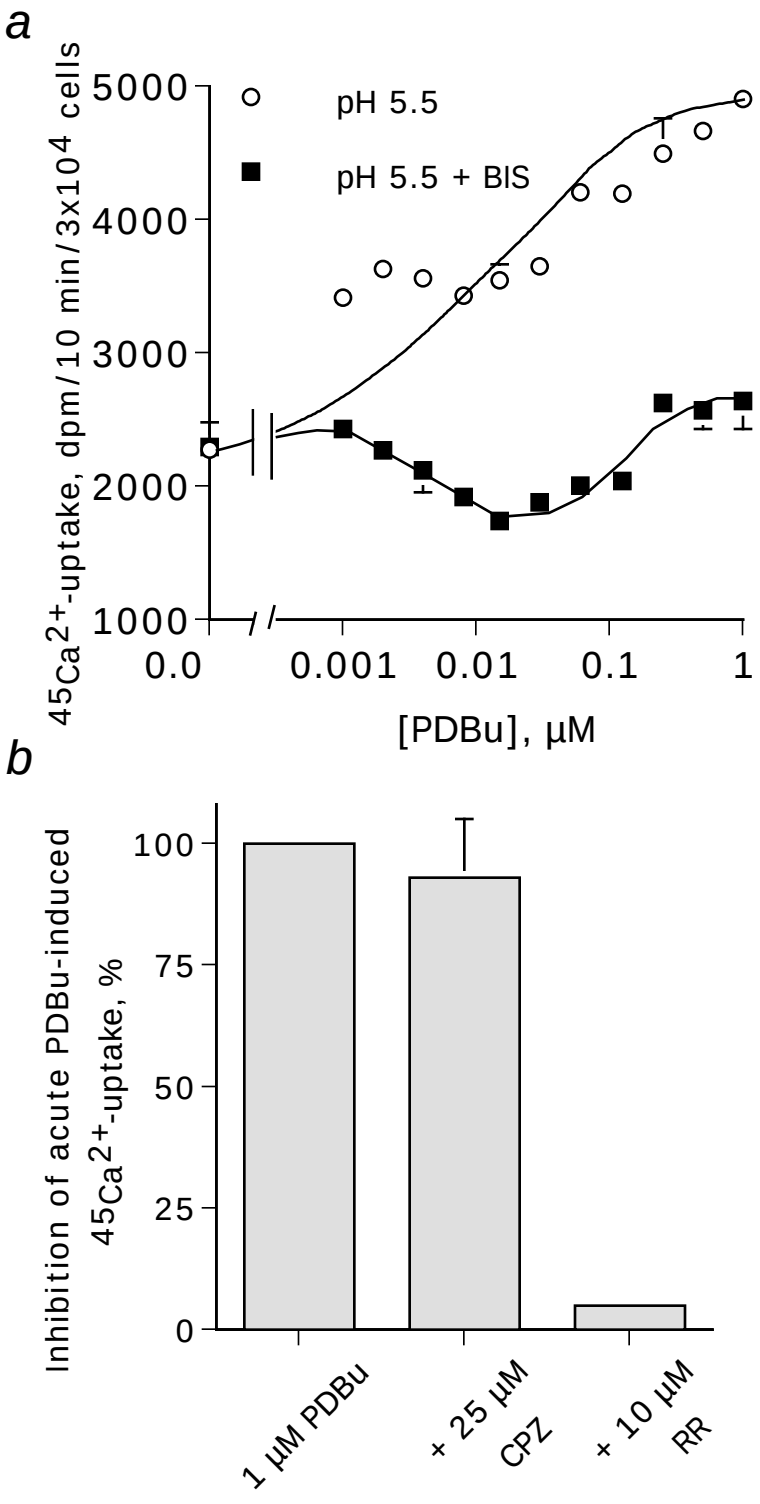
Fig. 7. VR1ε-HeLa. This stable cell line expresses recombinant VR1 in a different PKC milieu than NIH 3T3. The VR1ε-HeLa line was cultured and assayed as described for VR1ε-NIH3T3 cells. (*a*) VR1ε-HeLa cells showed ~10-fold activation of basal $^{45}Ca^{2+}$-uptake either induced with 10 μM CAP or 10 nM RTX at pH 5.5. (*b*) Expression of VR1 and PKC isozymes was determined with specific antibodies. In contrast to NIH 3T3, HeLa cells express PKCα, -β, -ζ and only weakly –δ, but not –ε, as determined with isozyme specific antibodies. However, only PKCα showed marked down-regulation due to chronic PDBu

treatment (2 μM, 24 hrs). The Western blot experiments were carried out and resulted in similar PKC isozyme and down-regulation patterns as shown.

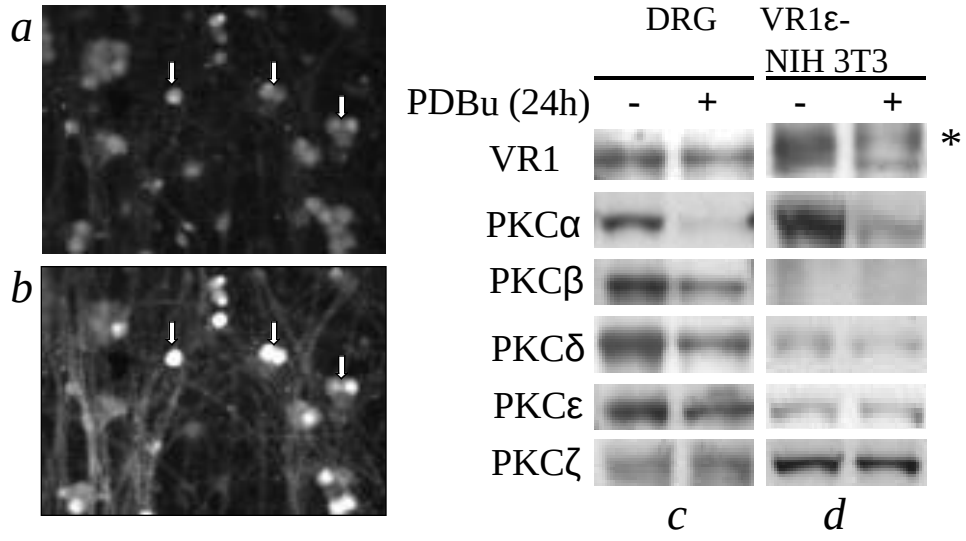
Fig. 8. To demonstrate the role of PKCα in activation of VR1, experiments were carried out with the same set of VR1-HeLa cells characterized in Fig. 7, which are deficient in PKCε. (*a*) Chronic PDBu inhibited, the acute effect of PDBu on VR1-mediated $^{45}Ca^{2+}$-uptake. (*b*) No inhibition on ANA-induced short-term $^{45}Ca^{2+}$-transport was seen with 24 hr PDBu exposure but a reduced affinity is obtained similar to that observed in VR1-NIH 3T3 cells. Note also the inverse concentration response in this cell line. Similar results were obtained in a second independent experiment.



Olah et al. Fig. 1



Olah et al. Fig. 2



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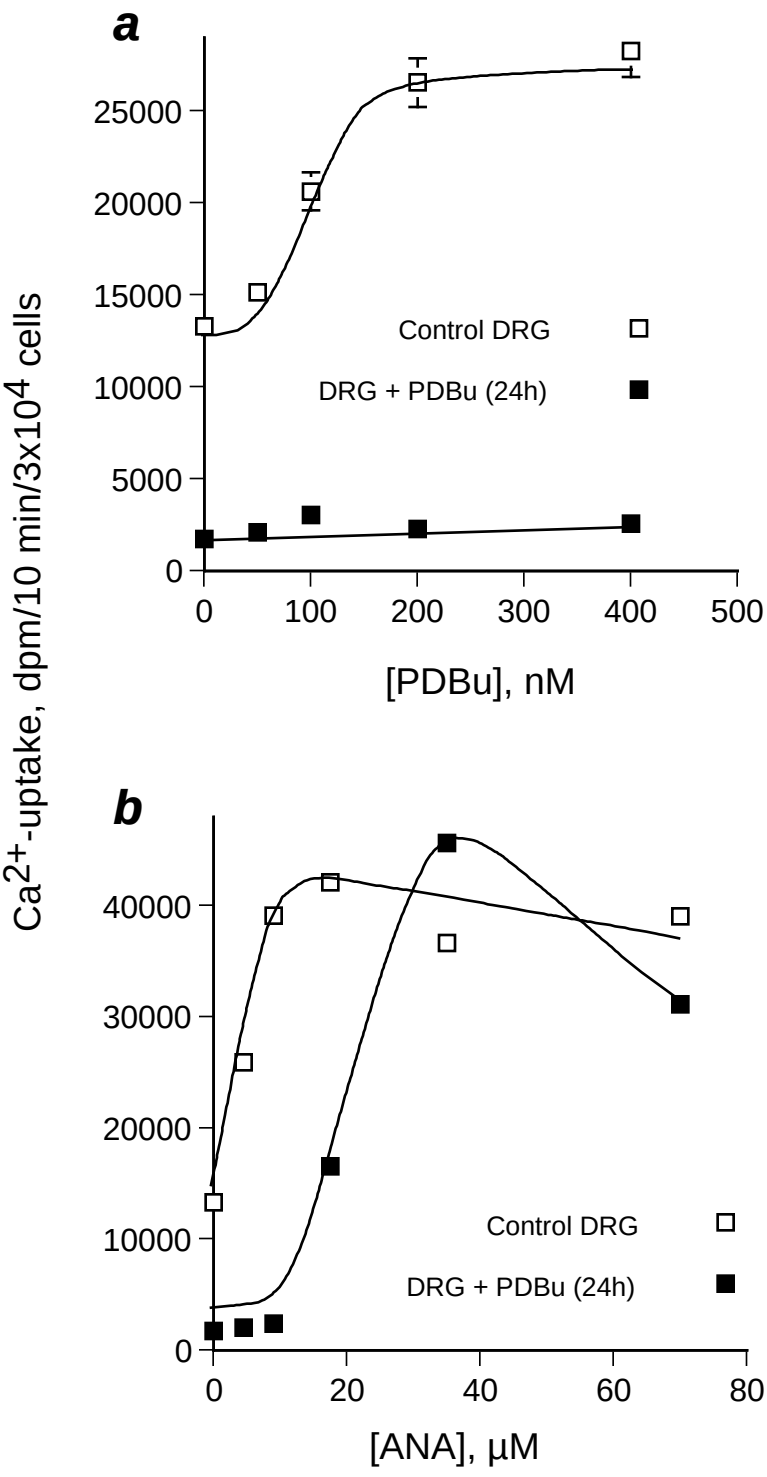

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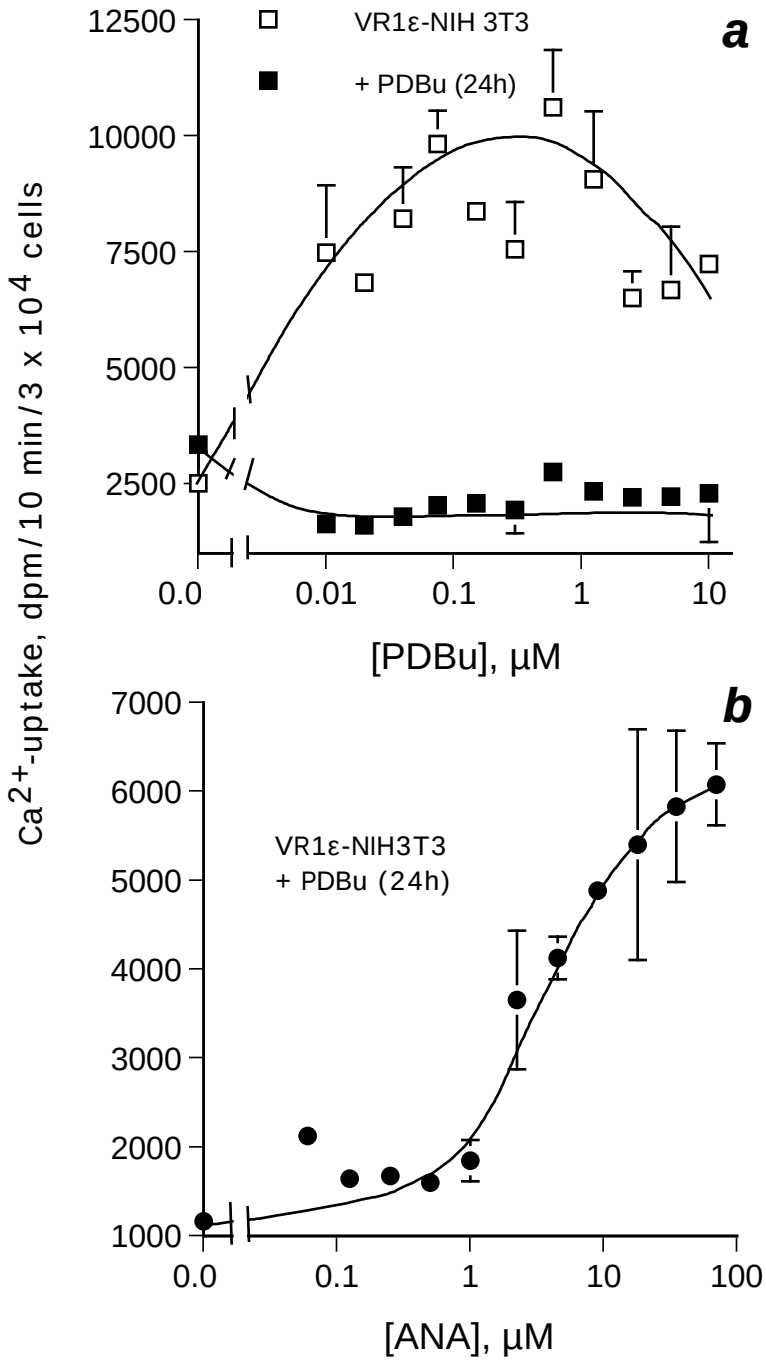

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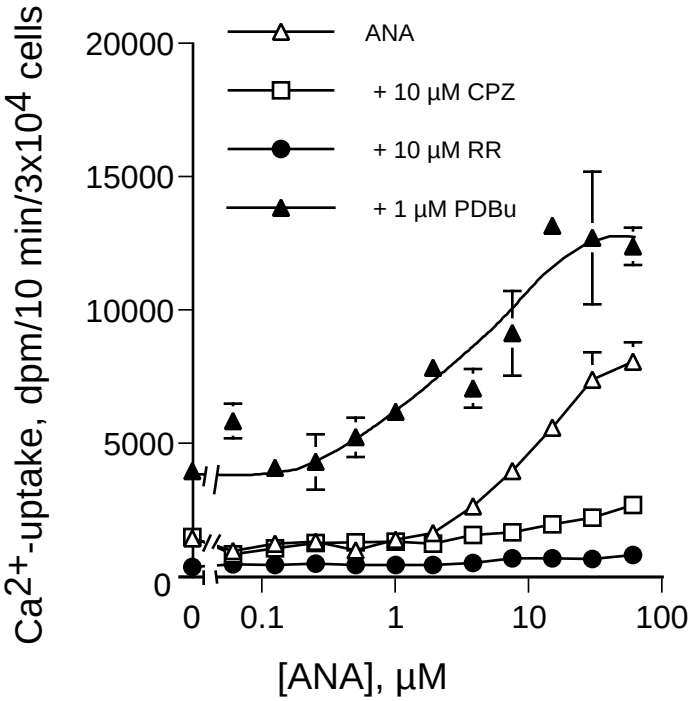

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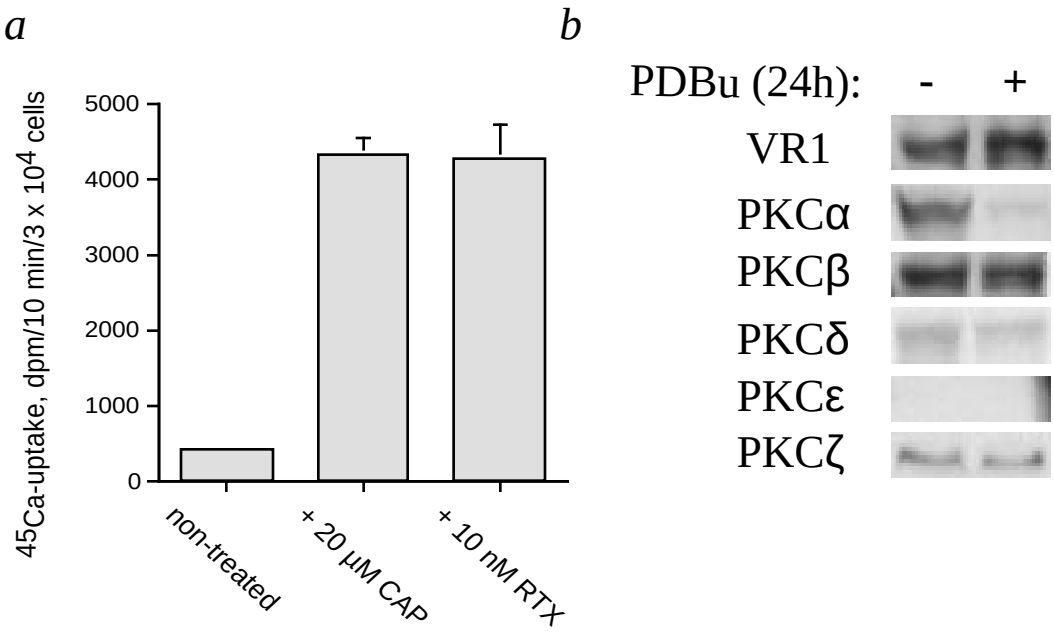

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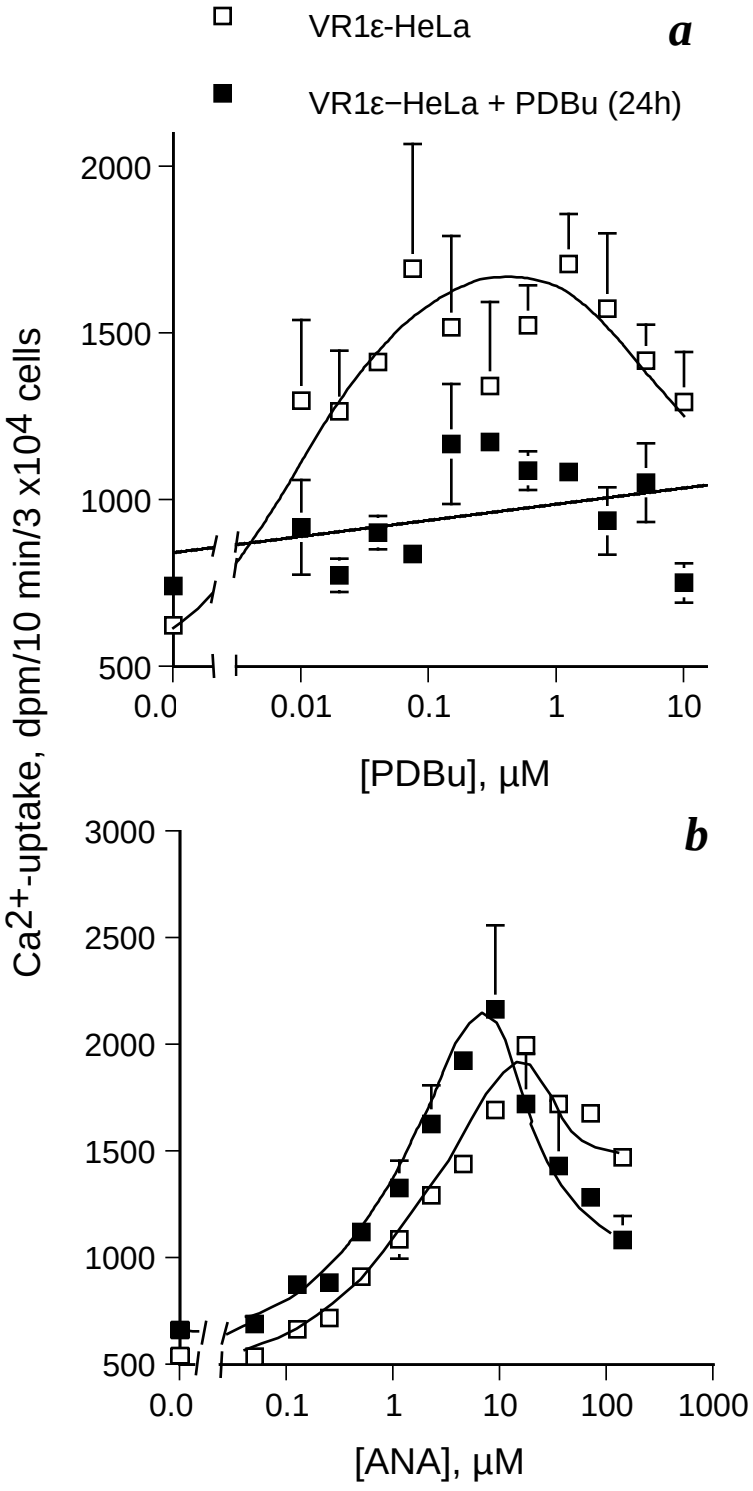

Olah et al. Fig. 8